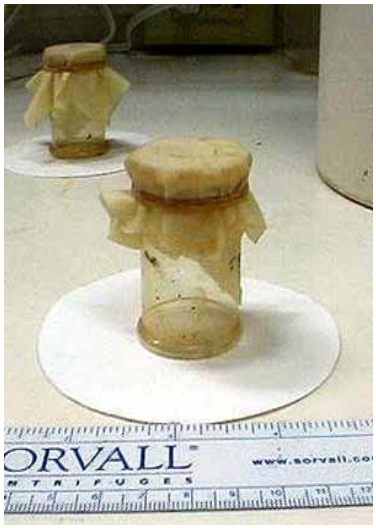
Microinjection Method for Anopheles Embryos V 2

Background:

The first method was developed by John R. (Randy) Clayton for injection of *Anopheles gambiae* embryos. It is the method used to obtain high frequency egg hatching and EGFP transient expression rates described briefly (Grossman et al. Germline transformation of the malaria vector, *Anopheles gambiae,* with the *piggyBac* transposable element. Insect. Mol. Biol. 2001). The primary distinctive feature is that embryos are injected under saline, the concentration of which is selected empirically to balance internal pressure so that resistance is sufficient that needle penetration is possible, yet low enough that oozing and needle backflow are minimized. The NaCl concentration can be adjusted as needed to achieve this end for each species.

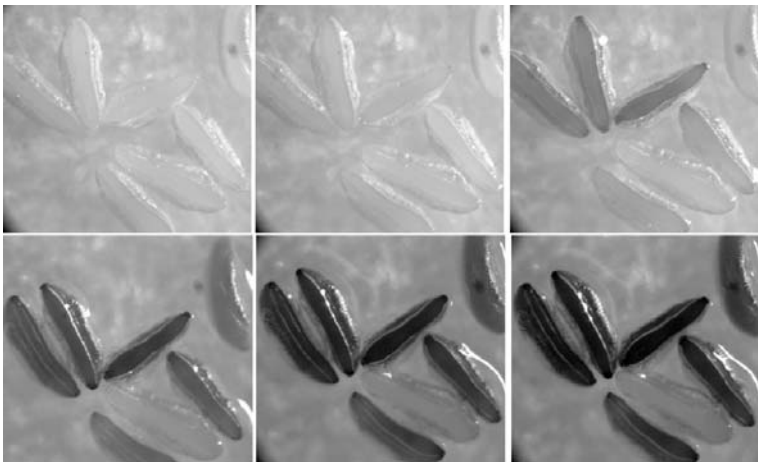


Adult female mosquitoes were bloodfed 3-5 days post-eclosion. Eggs were harvested 60-96 hours later by placing 10 to 15 adults in a transparent cylindrical container (~40 ml) that was open at one end and covered with rubber dental dam at the other (see left).

The container was slid over a single water-filled well on a cobalt blue ceramic 12-well depression plate (see right). The plate and container were then placed in the dark for 30 minutes for oviposition. The eggs were aged for 45 minutes at insectary conditions, (28°C; 80% humidity) after which time, the eggs were medium gray.

Alignment and injection were carried out at room temperature (approx. 24^{o}C). Eggs were transferred from the depressions to a glass slide with a fine paintbrush, where they were aligned with the dorsal (flattened, concave) surface facing up. The anterior ends were aligned in 25 mM NaCl against a strip of reduced-fiber filter paper. When 25 to 35 eggs had been aligned on a slide, the filter paper was removed by tugging it away sharply, so as not to disturb the alignment of the embryos. Eggs were then permitted to desiccate slightly. A glass coverslip with a piece of pre-cut double-sided adhesive was pressed gently against the eggs' dorsal surface and immediately inverted and covered with 25 mM NaCl solution to prevent drying and placed in a humid box at room temperature until injection. Getting the embryos to stick to the tape is the most difficult part of the above procedure. They cannot be too wet - in which they do not stick - or too dry - in which case they die.

Figure Right: Egg darkening at 20 min intervals (top left to right to bottom) beginning approximately 15 m after oviposition. Most of the darker eggs in the lower two left panels are suitable for injection. Note that one egg did not darken at all and will not hatch. When eggs have fully darkened, they are more difficult to inject. (photographs courtesy of G. Labbe, U. Lyon/IAEA)



For both methods, quartz glass micropipettes (O.D. 1.0 mm ; I.D. 0.7 mm) were pulled with a Sutter P-2000 Micropipette puller. The program for the P-2000 was: HEAT: 650, FIL: 4, VEL: 40, DEL: 150, PUL: 157. However, conditions necessary to produce suitable needles may differ on your device. Micropipettes were loaded with Eppendorf Microloader tips.

Immediately prior to injection, more 25 mM NaCl was added to the coverslip. A large volume surrounding the embryos is desirable as it reduces distortion of the image. The coverslip was then attached to a glass slide with double-stick tape and placed on the stage. Embryos were injected on the ventral surface, near the posterior end, with the embryo turned at an angle of about 15-25 degrees. The horizontal angle of the needle varied, but was roughly 30 degrees from the plane of the stage. D. O'brochta's group at the Univ. Maryland reports successful transformation of *A. gambiae* by varying the procedure above by covering the embryos with halocarbon oil prior to injection (see Kim et al. Ectopic expression of a cecropin gene… J. Med. Entomol. 41:447-455, 2004). This method should provide better visibility of the needle flow rate.

Care should be taken to avoid injection into the periplasmic space but immediately anterior to the periplasmic space and posterior to the egg floats. Injections were carried out at 100X magnification. Immediately after all of the embryos on a slide had been injected, the coverslip was removed from the scope and slide and placed into a cup of reverse-osmosis/deionized sterilized (RO/DI) H20 at RT to recover. When all prepared coverslips from a cohort had been aligned and injected, they were placed in 50 ml RO/DI H20 in the insectary conditions to hatch. It is not necessary that the eggs float for hatching.

Alternative Method

There is an alternative method that is faster and requires less judgment than the ones above. Herve Bossin and Mark Benedict developed it in Vienna for *A. arabiensis*, but it should be useful for many mosquito species.

The trick is to use a very thin blotting membrane (e.g. type HA) against which one can align the eggs without any adhesive. (I suspect any membrane that is thin, hydrophilic and does not contain detergent would be fine e.g. a Southern blotting membrane, or if you think it might contain detergent, rinse it well before use.) The point is that they are fiber-free, non-woven, and very thin. When they are wet, they adhere to a microscope slide so that the embryos don't slide beneath the membrane. We cut it at an angle so that the posterior edge we want to inject is perpendicular to the needle. Blotter paper on top keeps the moisture appropriate. Appropriate means a very thin meniscus of water around the embryos. This method is very easy, results in good survival and at least high levels of transient expression. A diagram of the set up is shown below. NOTE: The needle will not be submerged in liquid, so you will need to keep sufficient back-pressure on the needle to keep it cleared and wet.

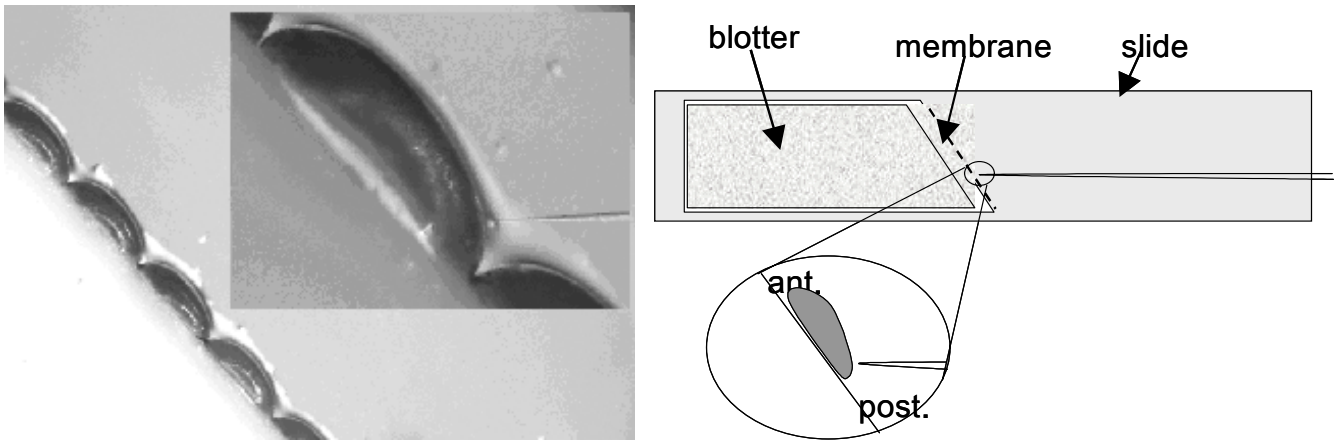


Figure Above: Eggs aligned for injection with a closeup of the needle approaching the injection area at a 90 degree angle (photograph courtesy of G. Labbe, U. Lyon/IAEA)

When aligning the embryos, roll them over so that the ventral side is upward and they will nest in the 90º niche between the membrane and slide nicely. Orient them all in the same direction. We find that using this method, they rarely move when being injected and can be rinsed off into a cup using water after injection.

Frequently Asked Questions

Q: Do you remove the chorion before injection as has been described in *Drosophila*?

A: No. endochorion removal in *anophelines* has not been accomplished. This is why the quality of the needles and turgor of the eggs is crucial.

Q. What does a good *A. gambiae* injection look like?

A: Larval hatch rates varied between 10% and 50% using either method. The most probable cause of this variation was the physical wounding of the embryo during injection. If the needle does not slide easily through the chorion of the egg during injection, then something is wrong. It is the ease of penetration that allows continuous injection without needle clogging or breakage.

Under good conditions, the needle will slide in and out of the egg with little effort. Slight resistance to penetration is apparent when entering the egg, and a small volume of yolk can sometimes be seen flowing into the tip of the capillary, only to be expelled immediately during injection. Although visibility is worse injecting under aqueous solution rather than halocarbon or mineral oil, a slight clearing of the yolk was often seen, even through the dark chorion. Injected eggs sometimes recoil and bulge briefly and slightly when a sufficient volume has been released into them and this is also a good sign so long as a minimal amount of yolk escapes from the wound site.

Q: I don't have a laser needle puller. Will this method work with boro- or aluminosilicate needles?

A: We have not attempted this. In principle, there is no reason why this method would not work with a softer glass but with frequent needle replacement. Quartz needles may simply allow a larger degree of error on the part of the person injecting. Aluminosilcate glass needles are preferable to borosilicate because of their greater hardness.

Q: Did you use a chorion hardening inhibitor?

A: No. We experimented with a number of inhibitors of the prophenoloxidase activation cascade (pNpGB, benserazide, PTU), but we found nothing which resulted in an increase in embryo injectability.

Q: *Do you bevel your needles?*

A: No. Non-beveled quartz is hard and sharp enough so that needles can be pulled and used immediately.

Q: *How do you prepare your DNA for injection?*

A: All DNA was prepared with a Qiagen Endo-Free kit and resuspended in injection buffer. It was then stored at -80oC until use. Immediately before use, it was thawed and spun through a 0.2 micron Millipore 1.5 mL filter to remove particulates. This latter measure (suggested by D. O'brochta) is simple and effective.

Q: What do you feed your hatching larvae?

A: We feed first-instar larvae two drops of 2% w/v baker's yeast on day two post-injection and another two drops on day four. Beyond day four, we feed as appropriate with our standard food mixture finely ground Koi Floating Blend.

Q: I thought anopheline eggs floated when they hatched. Aren't your injected embryos submerged when they hatch?

A: Yes. While *Anopheles* eggs do float for hatching, submerging eggs post-injection does not seem to have an strong effect on mortality relative to floating controls. In addition, this method avoids the large degree of mortality which was inflicted when attempting to remove the eggs from the adhesive surface of the tape.

Q: How hard do you press the coverslip down on the embryos when you're picking them up?

A: Delicately. Just hard enough to see that the eggs have come into contact with the tape and bulge slightly.

Q: What happens if I inject the embryos earlier than you describe?

A: Younger embryos are difficult to inject due to sensitivity to handling. Simply moving them early in development kills them.